Macrophage Stimulation and the Inflammatory Response to Asbestos

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Chrysotile fibers injected into the peritoneal cavity of mice elicit a cellular exudate. Macrophages appearing in this exudate produce high levels of the neutral protease, plasminogen activator, when compared with the resident peritoneal macrophage population. In contrast, the levels of lysozyme and two lysosomal enzymes are the same for the two macrophage types. The asbestos-induced macrophages producing the plasminogen activator appear to have descended from recently divided precursors. Low concentrations of anti-inflammatory glucocorticoids inhibit macrophage plasminogen activator synthesis.

Preliminary experiments indicate that different asbestos types induce hyperemia in skin, and also shorten the partial thromboplastin time of plasma and generate the release of kinins.

These observations could be interrelated and are suggested as representing some aspects of the inflammatory response of the host to asbestos exposure.

Introduction

Asbestos exposure appears to be associated with the development of a chronic granulomatous response (1) which leads to fibrosis surrounding the respiratory bronchioles and alveoli. It is apparent that additional biological assays are needed to analyze the biological events giving rise to asbestosis and, in some cases, neoplasms, and also to determine what structural features of asbestos fibers contribute to the development of these pathological changes. In this paper I present data on various aspects of the inflammatory response to asbestos fibers, viz., macrophage stimulation, quantitation of certain vascular changes, and interaction with plasma components. Apart from the obvious implications for the elucidation of the mechanisms of tissue damage and fibrosis, it could be relevant to asbestos-induced carcinogenesis as it is possible that the inflammatory response to asbestos is contributing to the progression of tumors.

Macrophages, a cell type present both in normal alveoli and in chronic inflammatory reactions, are often conspicuous in asbestos-induced lesions (2). When macrophages are stimulated *in vivo* or *in vitro,* they can secrete a number of biologically important

molecules. These include interferon (3), proteolytic enzymes (24), complement components (5), prostaglandins (6), colony-stimulating activity (7), and fibrogenic factors (8). It is likely that the initial event after inhalation of toxic particles such as asbestos is their attachment to and often phagocytosis by mononuclear phagocytes in the alveoli (9). Several workers have added different types of asbestos *in vitro* to both organ cultures and macrophages from several different species (10-16). In spite of the multitudinous conditions employed, it is possible to arrive at some general conclusions. When serpentine fibers (chrysotile A or B) are added to macrophages in the absence of serum, marked early cytotoxicity results. When macrophages are cultured in the presence of serum, exposure to asbestos fibers leads to delayed cytotoxicity, manifested after one or more days. Using lower concentrations of UICC chrysotile A(1-50 μg/ml), Davies et al. (17) showed that mouse peritoneal macrophages released lysosomal enzymes; this release occurred in the absence of any detectable cell death. We have studied another enzyme system found in mouse peritoneal macrophages, viz., plasminogen activator (18).

Peritoneal macrophages obtained from mice that have received inflammatory stimuli such as thioglycollate, endotoxin, mineral oil, etc., produce plasminogen activator (4, 19) whereas the resident peritoneal macrophages produce barely detectable

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levels. Hence there is a correlation between the production of this enzyme and an inflammatory response in the peritoneal cavity. We therefore decided to examine the *in vivo* and *in vitro* influence of asbestos fibers on the plasminogen activator levels of mouse macrophages. We have been able to demonstrate that UICC chrysotile Type B elicits macrophages into the peritoneal cavity of mice which produce high levels of this protease; *in vitro* addition of these same fibers to mouse peritoneal macrophages stimulates enzyme production. Preliminary experiments indicate that asbestos fibers increase the blood flow into intradermal sites of injection and also shorten the partial thromboplastin time of normal human plasma.

Materials and Methods

Reagents

Reagents were obtained as follows: fetal bovine serum (FBS) (Rehatuin FS, Reheis Chemical Company, Division of Armour Pharmaceutical Co., Phoenix, Arizona); asbestos, UICC Chrysotile Type B, a gift from Johns-Manville Corp.; Brewer thioglycollate medium (Difco Laboratories, Detroit, Michigan); soybean trypsin inhibitor, component VI, and bovine fibrinogen, fraction I (Miles Laboratories, Inc., Kankakee, Illinois); lactalbumin hydrolysate (Nutritional Biochemicals Corp., Cleveland, Ohio); outdated human plasma (New York Blood Center, New York, New York); polystyrene latex particles, 1.01 μm (Dow Diagnostics, Indianapolis, Indiana); dexamethasone (Sigma Chemical Co., St. Louis, Missouri); ^{125}I-sodium iodide, carrier-free (Schwarz/Mann Division, Becton, Dickinson & Co., Orangeburg, New York). The dexamethasone was dissolved in ethanol prior to dilution in phosphate buffered saline (PBS). Controls for the effect of the ethanol were never different from those of nontreated cultures. Endotoxin, *S. minnesota* lipopolysaccharide mR595 (S418), was a gift from Dr. O. Lüderitz, Max Planck Institut für Immunobiologie, Freiburg, West Germany.

Peritoneal Exudates

Peritoneal cells were obtained from NCS (Rockefeller) mice (25-30 g) or BDF* mice, 8-12 weeks old (Cumberland Farms) as previously described (4). The mice were injected intraperitoneally with 1 ml quantities of various irritants and the cells collected 4 days later. The cell yields after 4 days from asbestos-primed animals were 6-12 × 10^6 cells, increasing with the dose up to 100 μg, of which 30-40% were macrophages, 30-40% polymorphonuclear leukocytes (PMN) and 10-20% lymphocytes at this dose of asbestos. Thioglycollate-stimulated mice yielded 15-20 × 10^6 cells, consisting of 80-90% macrophages and 10-20% lymphocytes, while control mice gave 3-5 × 10^6 cells, of which 30-40% were macrophages and the remainder lymphocytes.

In Vivo Labeling of Asbestos-Induced Macrophages with Tritiated Thymidine (^{3}H-TdR)

Four mice were given 10 μCi ^{3}H-TdR (specific activity 10 Ci/mmole) intramuscularly 6 and 1 hr prior to an intraperitoneal injection of asbestos (300 μg), thioglycollate medium (1 ml), or normal saline. The ^{3}H-TdR injections were continued every 8 hr for the next 4 days. The peritoneal cells were harvested 4 days after the initial injections of the irritants in the usual manner (4).

Autoradiography

For the analysis of the *in vivo* radiolabeled peritoneal cells, the thioglycollate populations were plated at 2.5 × 10^5 cells in Dulbecco's medium (DB), supplemented with 10% fetal bovine serum that had been heat-inactivated at 56°C for 30 min (HIFBS), in Lab-Tek Chamber Slides (No. 4808); the asbestos and control (normal saline) cells were plated at 7.5 × 10^5 cells per chamber to ensure a comparable number of macrophages in the cultures. After 24 hr at 37°C, the cells were washed twice with PBS and DB + 0.05% lactalbumin hydrolysate (LH) was added. The conditioned media (CM) were collected, the cells washed twice with PBS and fixed with Carnoy's solution. The slides were dipped in Kodak Nuclear Track Emulsion (NTB2), dried, and stored at 4°C. After 21 days, autoradiographs were developed with Kodak D19 Developer for 5 min and fixed with Kodak Rapid Fixer. After developing, the cells were stained with Giemsa's stain. Since background levels in preparations from nonlabeled animals showed less than three grains per nucleus, all cells with three or more silver grains over the nucleus were considered as positive.

Fibrinolysis Measurements

The cells were plated on ^{125}I-fibrin-coated wells (Linbro Disposo Trays, FB 16-24 TC) for 24 hr in DB + 5% HIFBS (20). At this stage, soybean trypsin inhibitor (STI) (100 μg/ml) was added to prevent fibrinolysis. Fibrinolysis measurement was begun by washing the cells twice with PBS and placing them in DB supplemented with 5% acid-treated HIFBS (ATHIFBS). The HIFBS is acid-treated then neutralized to remove protease inhibitors during assay (20). Fibrinolysis was estimated by withdrawing

aliquots of medium and assaying them for radioactivity in a Packard Autogamma Scintillation Spectrometer. The plasminogen dependence of the fibrinolytic activity can be shown by assaying in the presence of ATHIFBS from which the plasminogen has been removed (21).

Plasminogen Activator Measurement

Alternatively, plasminogen activator can be assayed in the serum-free medium from the macrophages. Peritoneal cells were plated in DB + 5% HIFBS at the same density as above on tissue culture dishes (Falcon # 3001). After 24 hr, the cells were washed twice with PBS and incubated in serum-free conditioned medium consisting of DB + 0.05% LH (4). After 48 hr, the serum-free CM were collected. Serum-free CM were kept frozen at −20°C until assayed for enzymatic activity. The plasminogen activator in serum-free CM was assayed as described (4, 20). ^{125}I-Fibrin-coated wells of Linbro Disposo Trays were used, and the assay mixture contained 2 μg human plasminogen [purified from outdated human plasma according to Deutsch and Merz (22)], and 10 μl CM in 250 μl 0.1M Tris, pH 8.1. All experiments included appropriate controls for all reagents and media. No plasminogen-independent fibrinolysis could be detected when plasminogen was not included in the assay mixture. One unit of plasminogen activator is defined as the amount that stimulates the release of 10% of the initial radioactivity in 4 hr (4).

Protein

Protein was measured by the method of Lowry et al. (23) with the use of bovine serum albumin standard.

Results

Effects of Intraperitoneal Injection of Asbestos

Peritoneal Cell Population. It has previously been shown (18) that intraperitoneal injection of UICC chrysotile into mice increased the number of cells obtained by peritoneal washing and also affected the relative proportions of the different cell types present. Because of the relatively large proportion of PMNs (30-40% with 100 μg asbestos) present in these exudates, the cells were preincubated for 24 hr to allow for death of the PMNs (4) prior to the commencement of any experiment. After washing of the cells, at least 95% of the remaining cells were identified as macrophages. The asbestos-induced macrophages were larger and more vacuolated than those from PBS-injected mice.
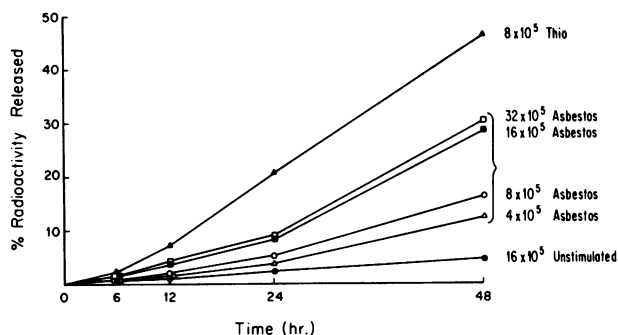

FIGURE 1. Plasminogen-dependent fibrinolytic activity from different numbers of peritoneal macrophages. For the *in vivo* priming, 100 μg asbestos was injected. Peritoneal cells on ^{125}I-fibrin-coated Linbro wells were cultured in DB+5% HIFBS+ 100 μg/ml STI for 24 hr, washed, and incubated in DB+5% ATHIFBS. Samples were aliquoted and counted for released radioactivity. The cell numbers are total peritoneal cell numbers plated.

Fibrinolytic Activity of Asbestos-Induced Macrophages. As shown previously, peritoneal exudate macrophages produce increasing levels of a plasminogen-dependent fibrinolytic activity as the *in vivo* asbestos dose increased (1-300 μg), and the enzyme was continually released for at least a 4-day period (18). Figure 1 shows that increasing fibrinolytic activity can be detected from asbestos-induced macrophages as the peritoneal cell number is increased from 4×10^5 until confluent macrophage cultures are obtained from approximately 2×10^6 peritoneal cells/well.

Peritoneal cell populations from thioglycollate- and PBS-primed animals are included for comparison. Again, there is continued increasing activity with time over the 48 hr assay period. Owing to the lower percentage of macrophages in an asbestos-induced exudate compared to a thioglycollate-induced population, cell confluence in the tissue culture wells occurred at approximately 2×10^6 peritoneal cells from asbestos-induced populations and 8×10^5 for the thioglycollate-induced exudate. It has previously been demonstrated that almost all of the fibrinolytic activity is plasminogen-dependent (18). In Table 1, a comparison is made between as-

Table 1. Fibrinolytic activity of induced peritoneal macrophages.[a]

Macrophages	Radioactivity released at 24 hr, %
Unstimulated (PBS)	1.4
Asbestos, 500 μg	52.9
Latex, 500 μg	3.0
Endotoxin, 30 μg	4.9

[a]2×10^6 peritoneal cells (4 day exudates) were plated for 24 hr on ^{125}I-fibrin. After washing in PBS, DB + 5% ATHIFBS was added to the cells and the plasminogen-dependent fibrinolytic activity monitored by counting released ^{125}I.

bestos and other agents in their ability to induce fibrinolytically active cells.

Asbestos is quite potent at eliciting "active" macrophages, in contrast to another nondigestible particle, latex.

Enzyme Secretion by Asbestos-Induced Macrophages. We also reported (18) that asbestos-induced macrophages secreted at least 20 times more plasminogen activator than resident peritoneal macrophages, whereas the amount of lysozyme secreted by the two types of macrophages was the same. Likewise, no difference was observed between asbestos-stimulated and control macrophages in the secretion of two lysosomal enzymes, N-acetyl-β-D-glucosaminidase and β-galactosidase (18). Hence, the increased secretion of plasminogen activator by asbestos-induced cells is a relatively specific response and is not part of a general increase in lytic enzyme synthesis.

In vivo Labeling of Macrophages with Tritiated Thymidine (^{3}H-TdR). It was then decided to determine how many asbestos-induced macrophages had come from precursors which had undergone DNA synthesis during the 4 day incubation period. Mice were injected both just prior to the asbestos injection and also repeatedly for the 4 day period after this. Table 2 indicates that approximately 70% of the macrophages after plating had incorporated ^{3}H-TdR into the nucleus. This was approximately the same value obtained for the thioglycollate-induced macrophages, whereas the percentage of labeled macrophages for the saline-treated control animals was approximately 11%.

The higher proportion of labeled cells in the stimulated populations, combined with the much higher levels of plasminogen activator (PA) produced by these populations, make it appear likely that the cells actively secreting PA are in fact the cells which have descended from precursors which had recently undergone DNA synthesis. It could also be demonstrated that both ultra-violet irradiation and also methotrexate were able to inhibit the generation of stimulated macrophages (as judged by PA synthesis) into the peritoneal cavity after intraperitoneal administration of thioglycollate (unpublished observations).

Table 2. *In Vivo* labeling of macrophages with tritiated thymidine (^{3}H-TdR).[a]

Macrophages	Labeled cells, %
Unstimulated	11 ± 1
Thioglycollate	69 ± 6
Asbestos	72 ± 4

[a]For experimental protocol see Materials and Methods. Values are means of triplicate cultures with standard errors.

Table 3. Effect of dexamethasone on the secretion of plasminogen activator by macrophages from asbestos-primed mice.[a]

Macrophages (6 × 10⁶)	Cell protein/dish, μg	Secreted plasminogen activator, units/mg cell protein
Asbestos (100 μg)	350	960
Asbestos + 10⁻⁷ M dexamethasone	320	24

[a]24 hr after plating in DB + 5% HIFBS in 35 mm dishes (Falcon 3001), asbestos-stimulated macrophages were washed, and placed in DB + 0.05% LH in the presence or absence of dexamethasone. CM were collected 48 hr later.

Effect of Dexamethasone on Macrophage Fibrinolytic Activity. Low concentrations of certain glucocorticoids can inhibit the synthesis and the secretion of plasminogen activator from thioglycollate-stimulated macrophages (20). In Table 3, a similar reduction in enzyme secretion is obtained if asbestos-primed macrophages are administered dexamethasone *in vitro*.

Other Aspects of the Inflammatory Response to Asbestos Fibers

We have recently dissected and quantitated two parameters of the inflammatory response in rabbit skin to asbestos fibers, viz., increased hyperemia and vascular permeability (24). Six asbestos fibers including chrysotiles and amphiboles induce increases in both of these parameters. We have also been able to demonstrate that asbestos fibers can shorten the partial thromboplastin time of normal plasma and, depending on the charge of the sample, can also activate factor XII to generate kinin activity (25). Since both fibrin deposition and kinin action have often been suggested to play important parts in inflammatory responses, I would like to propose that such studies may also be useful in delineating how asbestos interacts with host tissue to generate a chronic inflammatory lesion.

Discussion

There appears to be an association between the injection of an inflammatory stimulus into the peritoneal cavity of mice and the appearance of macrophages into the cavity which produce higher levels of the neutral protease, plasminogen activator. UICC chrysotile fibers are also able to elicit stimulated cells into the peritoneal cavity and also to stimulate peritoneal macrophages *in vitro* to produce more plasminogen activator (18). There does not seem to be a similar association between inflamma-
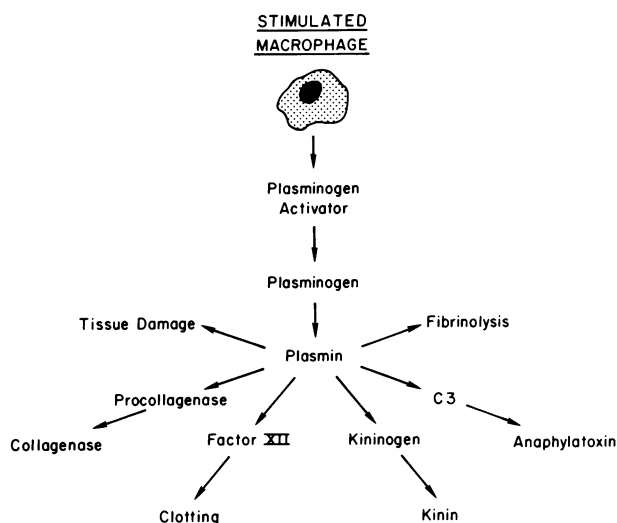
FIGURE 2. Some of the possible effects resulting from plasminogen activator release from macrophages.

tory stimuli and the levels of both lysozyme and also of enzymes of acid pH optima (18). Plasminogen activator and plasmin, the product formed after activation of the zymogen plasminogen, are both serine proteases which function optimally around neutral pH and hence at the pH of the extracellular milieu. Plasmin is likely to be important in inflammatory processes as it has been shown to activate certain enzyme cascades such as the complement (26), the clotting (27), and the kinin systems (28); in addition, plasmin is most likely to be involved in tissue destruction as it has been shown to degrade basement membrane and cartilage and also to activate a latent collagenase (29-31). Some of these possible plasmin-mediated events are depicted in Figure 2.

It is proposed that a part of the granulomatous response to asbestos fibers might be the interaction of asbestos with monocytes-macrophages resulting in the plasmin-mediated destruction of neighboring tissue and in the liberation of inflammatory mediators. In support of the proposal that the macrophage plasminogen activator-plasmin system might be relevant for the processes of chronic inflammation (particularly those associated with asbestosis), we have been able to demonstrate that low concentrations of anti-inflammatory steroids can inhibit macrophage plasminogen activator production. No such influence was found on lysozyme synthesis nor on lysosomal enzyme levels (18).

I propose that the mouse peritoneal macrophage system is a convenient model system to understand asbestos-macrophage interactions. However, it would also seem important to test the interaction with macrophage cell types that are of more direct interest and are most likely to be in contact with

asbestos, viz., lung macrophages. Perhaps alveolar macrophages from workers exposed to asbestos fibers would produce more plasminogen activator than the non-exposed counterparts. Since a single cell assay is available for plasminogen activator detection (20), it might be possible to analyze such cells at the single cell level. Peritoneal macrophages might also have a role in asbestos-associated neoplasms because of the occurrence of peritoneal mesotheliomas.

As described previously (18), asbestos fibers can stimulate mouse macrophages in vitro to produce more plasminogen activator when added to the cells in culture. The mechanisms of interaction of asbestos fibers with macrophages are unknown, although phagocytosis is possibly important. This question can only be resolved if more defined fibers are used and if cells exposed to asbestos in vitro are examined for intracellular fibers by such techniques as electron microscopy. One observation which could be relevant for agents such as asbestos is that persistent phagocytic stimuli seem to be able to cause macrophages to produce plasminogen activator continually as opposed to digestible materials (19). This could have serious consequences, as there would be no obvious way of reducing plasminogen activator synthesis, particularly as the nondegradable asbestos could be released from dying cells and taken up by another cell. It would be worth exploring the possibility of modifying asbestos fibers so that they cannot cause macrophages to produce more plasminogen activator.

While I do not wish to imply that stimulation of the macrophage plasminogen activator-plasmin system is the only critical event involved in the establishment of the chronic lesions of asbestos, it could represent an important feature. The structural properties and the inflammatory host interactions that enable asbestos fibers to elicit macrophages into the peritoneal cavity are unknown at the present. Some preliminary experiments designed to examine such questions were mentioned above and are now discussed.

The early vascular changes induced by asbestos interaction with tissues have virtually been unexamined, although Pelfrene (32) recently elicited vascular modifications in the hamster cheek pouch. We have quantitated both the increased hyperemia and vascular permeability elicited in rabbit skin to a series of asbestos and related fibers. It is now becoming clear with other irritants that increased initial blood flow in response to irritation (and damage) can be a controlling influence on other vascular changes (33). As hyperemia, vascular permeability and leucocyte extravasation can now be measured in various organs of several species, it is suggested that

it might be appropriate to monitor these parameters at tissue sites with asbestos involvement.

The fact that asbestos fibers can shorten the partial thromboplastin time (PTT) and, depending on the charge, can also generate kinin activity from normal human plasma, may be partial explanations, at least, for the relationship between asbestos inhalation, lung disease and possibly neoplasia. This is because both chronic activation of the coagulation system leading to the deposition of fibrin and the generation of vascular mediators, and also the production of kinins, are strongly implicated in the establishment of chronic inflammatory lesions and eventually to tissue destruction (34, 35).

In conclusion, I would like to suggest that I have presented data relevant to some of the early vascular events associated with asbestos exposure and also relevant to the role of inflammatory macrophages responding to the asbestos stimulus.

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